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PRINCIPAL INVESTIGATOR: Dr. Natasa Strbo

CONTRACTING ORGANIZATION: University of Miami
Coral Gables, FL 33146

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

The innovative approach taken by our laboratory, relies on secreted gp96-Ig chaperoning antigenic proteins that are efficiently taken up by activated APCs and cross presented via MHC I to CD8 CTL, thereby stimulating an avid, antigen specific, cytotoxic CD8 T cell response. Here we developed malaria vaccine that relies on secreted gp96-Ig chaperoning Plasmodium falciparum antigenic sporozoite proteins CSP and AMA1. The generation of a powerful, cytotoxic anti sporozoite CD8 CTL response by the vaccine is expected to provide prophylactic immunity for malaria by removing infected liver cells before sporozoites can replicate and spread to the erythrocyte stage causing parasitemia.

In the third year, we completed all proposed mouse immunogenicity experiments that addressed the effect of secondary 293-gp96-Ig PfAMA1-PfCSP immunization and induced memory responses as well as we compared the immunogenicity of the 293-gp96-IgPfAMA1-PfCSP vaccine to the immunogenicity of NMRC-M3V-D/Ad-PfCA vaccine. We found that gp96-Ig vaccination provided stronger antigen specific CD8 T cell responses, systemically as well as in the liver. We have also completed manufacturing of GMP-grade vaccine material for use in non-human primate studies.

15. SUBJECT TERMS

Malaria, Plasmodium Falciparum, circumsporozoite protein (CSP), apical membrane antigen-1, vaccine (AMA1), heat shock proteins, gp96-Ig, cytotoxic T cells, cell mediated immunity

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1. INTRODUCTION:

We have previously shown that cell-based vaccines secreting heat shock protein gp96-Ig (for short from here on: **gp96**) are safe for use in humans and represent the most efficient vaccine approach studied to date for stimulating multi-epitope specific cytotoxic T cells. In the proposed studies, we will adapt this vaccine approach to stimulate cytotoxic T cells against malaria antigens and investigate the optimal vaccination route to target these T cells to the liver. To accomplish these studies, we are collaborating with experts in the malaria vaccine field, Capt. Eileen F. Villasante, M.D., Ph.D., Head Malaria Department Infectious Diseases Directorate at Naval Medical Research Center and Lt. Kimberly A. Edgel, PhD, Deputy Head, Malaria Department Infectious Diseases Directorate at Naval Medical Research Center. By conducting head-to-head studies to another promising malaria vaccine, these studies will help to set clinical priorities based on the most effective pre-clinical data in animal models.

2. KEYWORDS:

Malaria, *Plasmodium Falciparum*, circumsporozoite protein (CSP), apical membrane antigen-1, vaccine (AMA1), heat shock proteins, gp96-Ig, cytotoxic T cells, cell mediated immunity

3. OVERALL PROJECT SUMMARY:

The goal of our project is to combine the *Plasmodium falciparum* (Pf) antigens circumsporozoite protein (CSP) and apical membrane antigen-1 (AMA1) with a novel method of immunization that is based on the gp96-Ig vaccine platform to enable production of a strong, protective, cell-mediated immunity (CMI) response (interferon gamma [IFN- γ]-positive CD8+ cytotoxic T cells).

This will be accomplished through three specific aims: (1) construction of the 293-gp96-Ig^{PfAMA1-PfCSP} and 293^{PfAMA1-PfCSP} vaccine cell lines; (2) determination of the safety and immunogenicity of the 293-gp96-Ig^{PfAMA1-PfCSP} vaccine in mice; and (3) determination of the safety and immunogenicity of the 293-gp96-Ig^{PfAMA1-PfCSP} vaccine in rhesus macaques.

Summary of Current Objectives: During the last year we have been intensively working on the experiments related to:

Specific Aim 2b: Determine non-inferiority at the time of the vaccine 'memory' response by comparative vaccination with the 293-gp96-IgPfAMA1-PfCSP vaccine versus the NMRC-M3V-D/Ad-PfCA vaccine.

Specific Aim 3, Task 3a: Manufacturing of GMP-grade vaccine material for use in non-human primate studies (Task 3b).

Summary of Results:

In our previous studies during past two years, we generated 293-gp96-IgPfAMA1-PfCSP vaccine cell line and demonstrated that 293-gp96-IgPfAMA1-PfCSP vaccine cell line is immunogenic in the mouse model. In addition, we found that subcutaneous route of vaccination induces dramatic increase in the liver-infiltrating CD8+ T cells. Importantly, the magnitude of malaria antigen-specific CD8+ T cell responses is believed to be the best measure of immunity targeting the hepatic stages of infection and the failure of the RTS,S vaccine to stimulate CD8+ cytotoxic T cell immunity was a significant weakness in the approach. Our findings are strongly supportive of the novel gp96-Ig malaria vaccine as unique systemic and liver-homing, sporozoite specific CD8 CTL vaccine strategy.

Following successful completion of our milestones, in the year three we completed all experiments under Specific Aim 2b (side by side comparison of memory responses induced by 293-gp96-IgPfAMA1-PfCSP vaccine cell and NMRC-M3V-D/Ad-PfCA) and here is the **Summary of memory response results:**

We have identified, (Figure 1) that subcutaneous route of vaccination together with intraperitoneal can induce the highest frequencies of PfAMA1 and PfCSP specific CD8 T cells. In addition to induction of antigen specific CD8 T cell responses systemically (spleen), subcutaneous route of vaccination was also the superior in induction of antigen specific CD8 T cell responses in the liver (Figure 1). Evaluation of liver-specific accumulation of PfCSP and

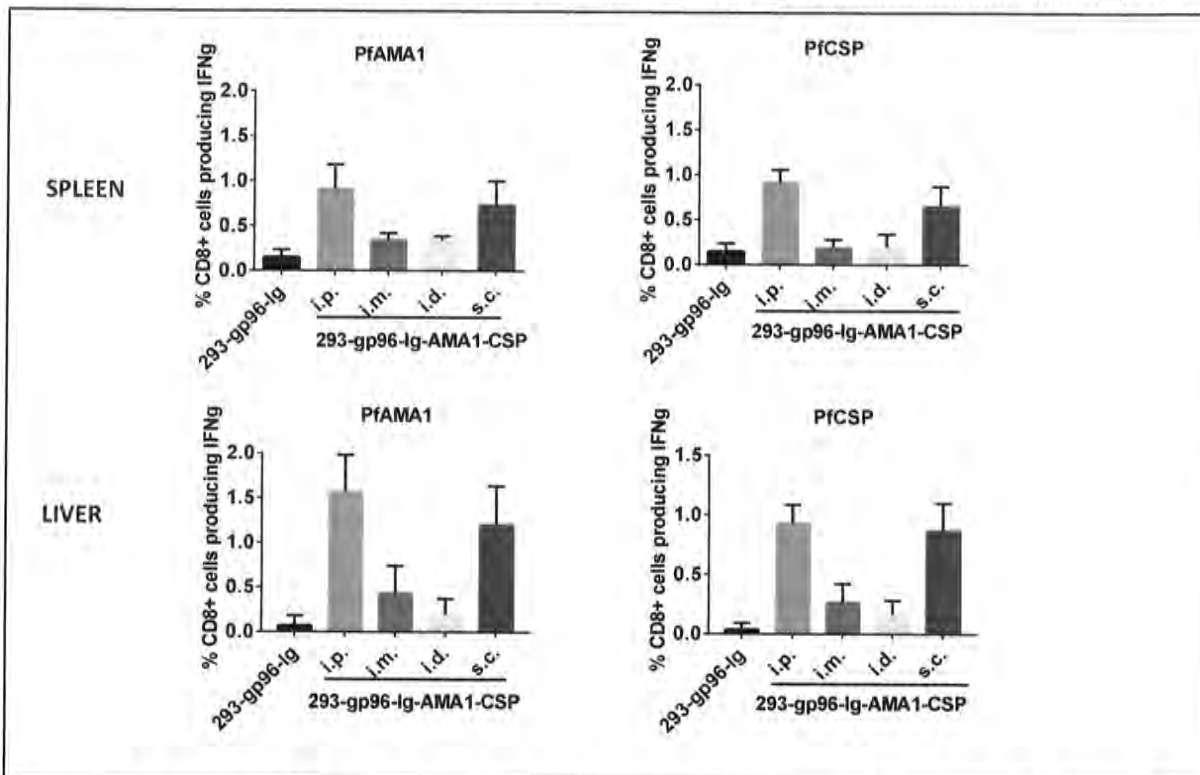


Figure 1. 293-gp96-Ig-PfAMA1-PfCSP vaccination delivered by subcutaneous and intraperitoneal route induce the highest frequencies of PfAMA1 and PfCSP specific CD8 T cell responses in the spleen and liver. One million 293-gp96-Ig-PfAMA1-PfCSP cells were injected in B6 mice by intraperitoneal (i.p.), intra muscular (i.m.), intradermal (i.d.) and subcutaneous (s.c.) route. Five day later mice were sacrificed and frequency of PfAMA1 and PfCSP specific CD8 T cell responses in the spleen (SPL) and liver were measured after in vitro peptide stimulation by intracellular cytokine staining (ICS) assay and analyzed by flow cytometry. Control mice were injected with one million of 293-gp96-Ig cells by i.p. route. Bar graph shows percentage of PfAMA1 and PfCSP specific CD3+CD8+ cells that produce IFNγ

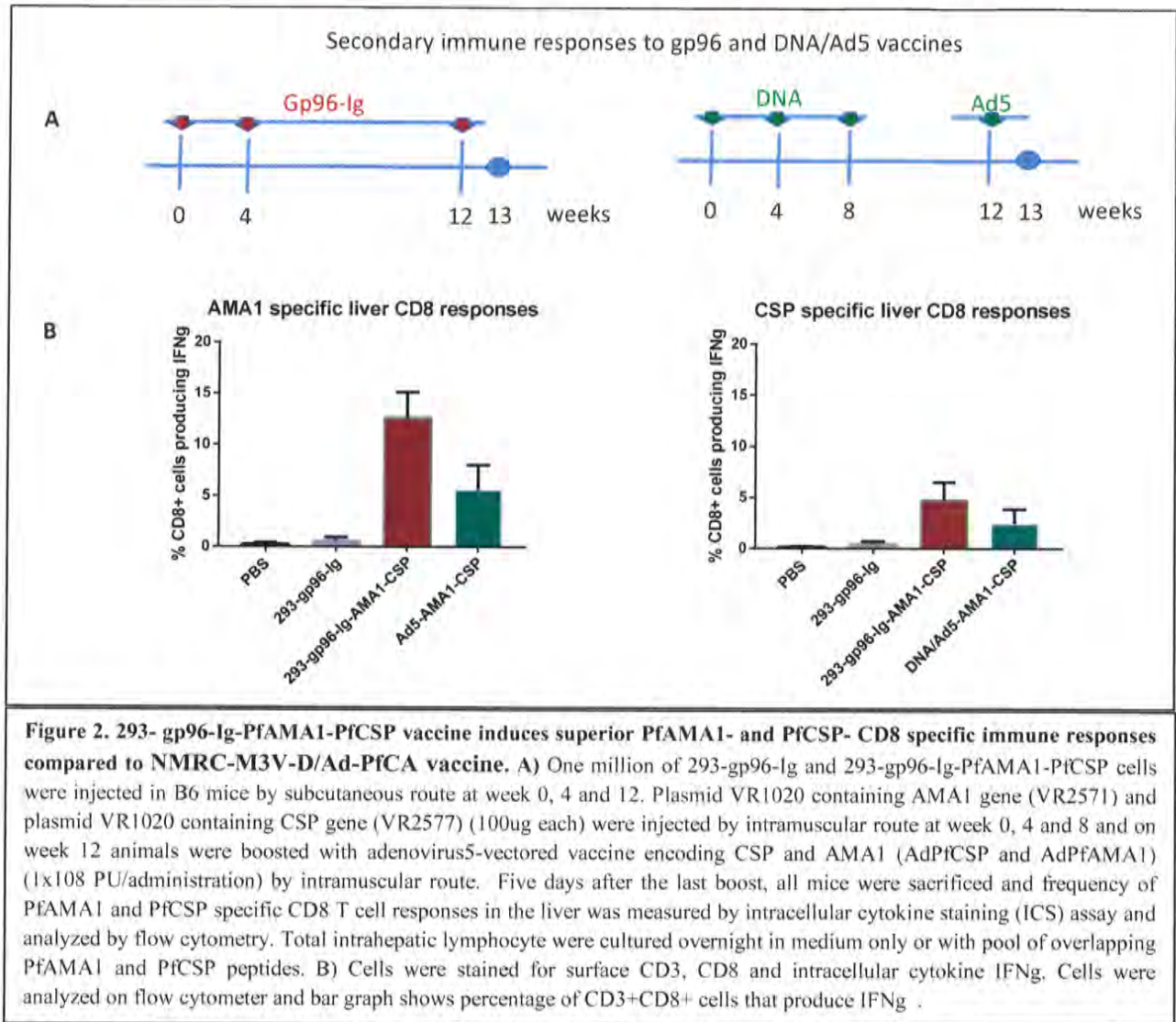
PfAMA1-specific CTL is therefore an important milestone in determining the optimal immunization route to be examined in rhesus macaques.

Furthermore, since the translation of intraperitoneal route of vaccination to the clinical settings could present a big hurdle, we decided to pursue subcutaneous delivery of gp96-Ig vaccine in all future immunogenicity experiments (mouse and nonhuman primate in vivo experiments).

Accordingly, we performed mouse secondary immunization studies using the optimal immunization route from primary immunization studies, subcutaneous route, in order to measure the induction of vaccine-induced memory responses. We deliver gp96-Ig subcutaneously and NMRC-M3V-D/Ad-PfCA vaccine intramuscularly. We treated mice with either the gp96-Ig vaccine or the NMRC-M3V-D/Ad-PfCA vaccine on day 48 after the indicated 'priming' regimens (Figure 2 A) for each treatment. The memory response was

measured on day 5 following the vaccine 'boost', among liver-infiltrating, mesenteric lymph node and splenic T cells (results shown only for liver-infiltrating T cells Figure 2B).

Prior vaccination, production of gp96-Ig was measured by established ELISA protocol using supernatant from 1×10^6 cells that are cultured in 1 ml for 24h. Western blotting with anti-PfCSP and anti-PfAMA1 as primary antibody and anti IgG-HRP as secondary labeled



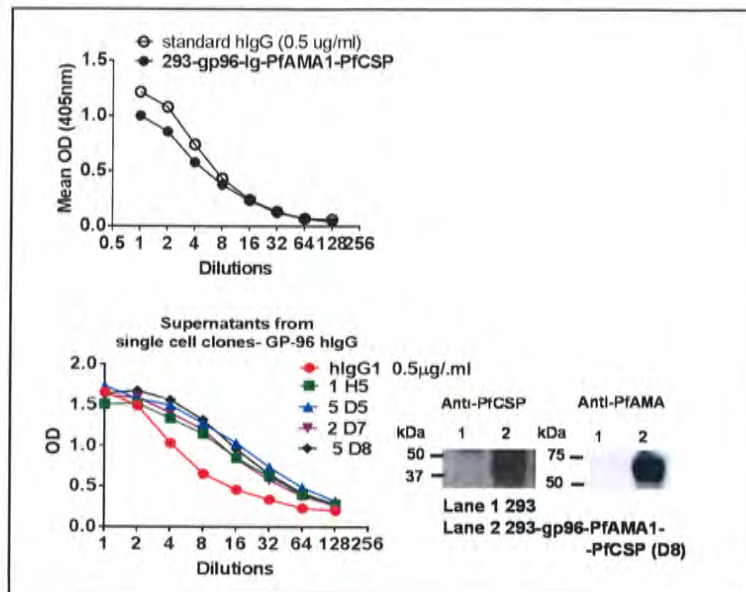
antibody confirmed expression of PfCSP and PfAMA1 protein.

B6 mice were vaccinated with 293-gp96-Ig^{PfAMA1-PfCSP} by subcutaneous route and with DNA/AD5 vaccine by intramuscular route according to the vaccine regiment shown on Figure 2A. We also used Mock controls: 293-gp96-Ig. Five days after the last boost, mice were sacrificed, spleen, mesenteric lymph nodes and liver were collected and AMA1 and CSP specific CD8 T cells responses were measured by intracellular cytokine staining (ICS) assay. Two pools of overlapping CSP and AMA1 peptides that we used to stimulate lymphocytes obtained from spleen and liver *in vitro* and measure the production of IFN- γ and TNF- α (data not shown), by intracellular cytokine staining and flow cytometry to assess the specificity of the gp96-Ig induced CD8 T cells. In animals that were vaccinated with 293-gp96-Ig^{PfAMA1-PfCSP}, we observed the highest frequency of PfCSP and PfAMA1 specific CD8+ T cells (Figure 2 B) while their frequencies were at the background level for the animals that received Mock

control (293-gp96-Ig). We found slightly higher frequency of PfAMA1 specific CD8+ T cells that produce IFN γ compared to PfCSP specific CD8+ T cells (Figure 2 B).

Since the objective of the Task 2b was to find if the 293-gp96-Ig PfAMA1-PfCSP vaccine is non-inferior at the time of the vaccine memory response by head-to-head comparison with NMRC-M3V-D/Ad-PfCA before advancing to Specific Aim 3, we set up experiments shown in Figure 2A and B to address this question. We found that the gp96-Ig vaccine represent superior vaccination regiment for induction of AMA1 and CSP specific CD8 T cell memory responses.

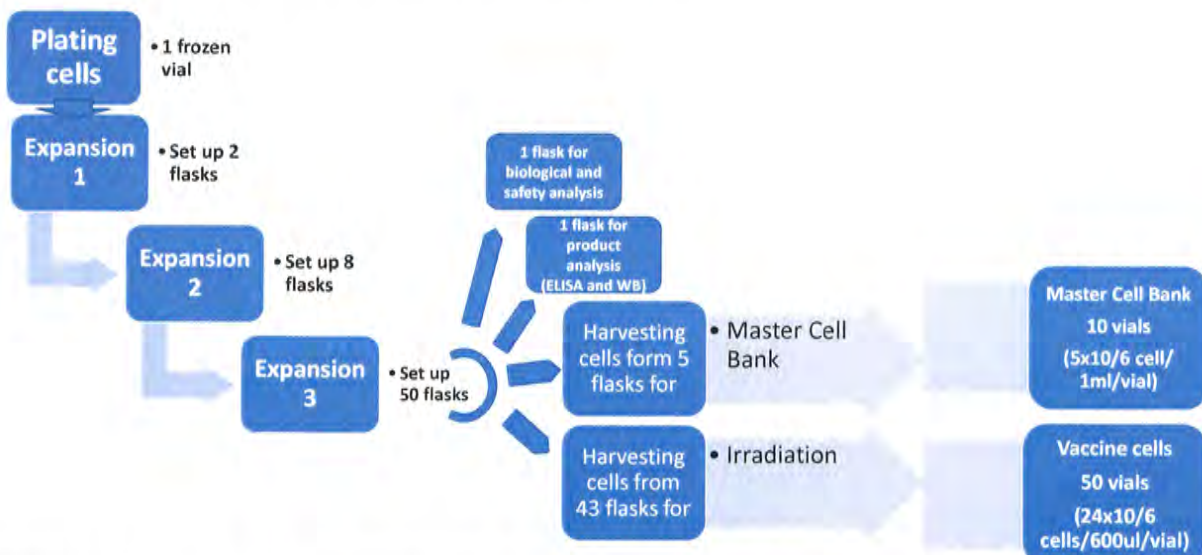
Summary of gp96-Ig vaccine manufacturing



To facilitate progression from these studies to nonhuman primate studies (Task 3b) and eventually to IND and Phase I clinical testing of

Figure 3 Single cell cloning of gp96-Ig malaria vaccine. : One million of 293-gp96-Ig-PfAMA1-PfCSP cells and different single cell clones (H5, D5, D7, D8) were plated in 1 ml for 24 h and gp96-Ig production in the supernatant was determined by ELISA using anti-human IgG antibody for detection with mouse IgG1 as a standard. **Western blot of 293- gp96-Ig-PfAMA1-PfCSP.** 293- gp96-Ig-PfAMA1-PfCSP cells (single cell clone D8) were analyzed by SDS-PAGE and Western blotting with anti-PfCSP and anti-PfAMA1 as primary antibody and anti IgG-HRP as secondary labeled antibody

Process flow diagram for producing biological product



293-gp96-Ig^{PfAMA1-PfCSP}, we will utilize GMP-grade vaccine material. Thus, the first objective

of specific aim 3 was to manufacture GMP-grade vaccine material. Vaccine cells that were generated in our laboratory have been significantly improved regarding the gp96-Ig production (Figure 3). In order to establish the vaccine cell line that can be manufactured in cGMP facility, we went through the process of single cell cloning to select for the vaccine cells with the highest production of gp96-Ig as well as AMA1 and CSP protein expression (Figure 3). We developed vaccine cell 293-gp96-Ig^{PfAMA1-PfCSP} that produced app. 2µg/ml gp96-Ig in a standardized ELISA assay (Figure 3. Clone H5, D5, D7, D8).

The highest producing clone was then transferred to the ISCS University of Miami cGMP facility and flow diagram of the manufacturing procedure is shown. Total of 50 vaccine vials was produced as well as master cell bank that can be used for future expansion and production.

Summary of Progress and Accomplishment with Discussion:

We demonstrated that 293-gp96-IgPfAMA1-PfCSP vaccine cell line is safe and immunogenic in the mouse model.

We preformed head-to-head immunogenicity comparison to the protective, T cell immunity-based NMRC-M3V-D/Ad-PfCA vaccine (DNA prime/adenovirus boost regimen). We demonstrated that 293-gp96-IgPfAMA1-PfCSP vaccine cell line is immunogenic and can induce superior memory responses compared to NMRC-M3V-D/Ad-PfCA vaccine.

The criteria on which a 'go' or 'no-go' decision is reached was based upon the relative differences between murine CD8+ T cell specific immune responses observed following immunization with NMRC-M3V-D/Ad-PfCA as compared to 293-gp96-IgPfAMA1-PfCSP vaccine. Specifically, this selection is based dually upon the percentage of IFNγ positive CD8+ T cells following re-stimulation with overlapping peptide libraries from the PfCSP and PfAMA1 antigens as detected by flow cytometry assays. **Superior treatment difference as measured by CD8+ IFNγ responses comparing 293-gp96-IgPfAMA1-PfCSP and NMRC-M3V-D/Ad-PfCA for CSP or AMA1 met the "go criteria" for moving forward to NHP studies (Specific Aim 3b).**

Our findings are strongly supportive of the novel gp96-Ig malaria vaccine as unique systemic and liver-homing, sporozoite specific CD8 CTL vaccine strategy.

Following successful completion of our final "go criteria" milestones, we proceeded to experiments under Specific Aim 3 (manufacturing of gp96-Ig vaccine) and we have successfully completed all of the proposed tasks.

Our collaborators, Capt. Eileen F. Villasante, M.D., Ph.D., Head Malaria Department Infectious Diseases Directorate at Naval Medical Research Center and Lt. Kimberly A. Edgel, PhD, Deputy Head, Malaria Department Infectious Diseases Directorate at Naval Medical Research Center are in the process of finalizing the IACUC protocol for nonhuman primate immunogenicity studies (Task 3b).

4. KEY RESEARCH ACCOMPLISHMENTS:

We demonstrated that 293-gp96-IgPfAMA1-PfCSP vaccine is non-inferior at the time of the vaccine memory response by comparative vaccination with 293-gp96-Ig PfAMA1-PfCSP vaccine versus the NMRC-M3V-D/Ad-PfCA.

We generated vaccine cells that produce/secrete high level of gp96-Ig and plasmodium falciparum antigens (Pf) AMA1 and CSP and finished manufacturing of GMP-grade vaccine material for use in non-human primate studies (Task 3b).

5. CONCLUSION:

Our approach to vaccine development is to develop a multi-antigen malaria vaccine by generating high levels of multi-epitope, plasmodium-antigen specific CD8 cytotoxic T lymphocytes, mimicking the radiation attenuated whole parasite. Our experience documents that the cell based gp96-Ig approach is highly effective in generating high levels of antigen specific CD8 CTL which is effective in stimulating high-frequencies of poly-antigen specific CTL in both human cancer patients and SIV-specific CTL in rhesus macaques and which is safe. We adapted this vaccine strategy to malaria, and we transfect HEK-293 cells with the Plasmodium Falciparum circumsporozoite protein (PfCSP) and apical membrane antigen 1 (PfAMA-1) and with gp96-Ig and generated vaccine cells line 293-gp96-Ig^{PfAMA1-PfCSP}.

Our immunogenicity studies in mice were designed to enable a nonhuman primate immunogenicity studies. We have provide a head-to-head comparison to another promising malarial vaccine candidate, NMRC-M3V-Ad-PfCA and confirmed gp96-Ig superior induction of memory responses compared to DNA/Ad5 vaccine regiment.

The ultimate goal is to develop a universal vaccine that is highly effective and practical, which is in line with the DoD area of research interest.

Our findings are strongly supportive of the novel gp96-Ig malaria vaccine strategy as unique **systemic and liver-homing, sporozoite specific CD8 CTL vaccine strategy.**

6. PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS:

Poster presentation at

IMMUNOLOGY 2016™
MAY 13 - 17 | AAI ANNUAL MEETING | WASHINGTON STATE CONVENTION CENTER | SEATTLE, WA

Secreted heat shock protein gp96-Ig vaccine for malaria prophylaxis

L. Romero ^a, E.R. Podack ^a E.F. Villasante ^b, K.A. Edgel ^b, N. Strbo ^a.

^a Department of Microbiology and Immunology, Miller School of Medicine University of Miami, Miami, FL, 33136 and ^b Malaria Department, Infectious Diseases Directorate, Naval Medical Research Center, Silver Spring, MD 20910

7. INVENTIONS, PATENTS AND LICENSES:

Nothing to report

8. REPORTABLE OUTCOMES:

Nothing to report

9. OTHER ACHIEVEMENTS:

- We have developed and manufactured 293-gp96-IgPfAMA1-PfCSP cell line

10. **REFERENCES:**

Nothing to report

11. **APPENDICES:**

Nothing to report